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FOREWORD

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INTRODUCTION

Tropomyosin (TM) expression is altered in many transformed cell types (1-4). The present report documents the results of experiments analyzing the expression of TM isoforms in normal and malignant human breast epithelial cells. TMs are actin-binding proteins which help to regulate the Ca^{2+} sensitivity of contraction in muscle cells (5) and may regulate cell motility and intracellular vesicular transport among other processes, in non-muscle cells (6). Their expression is normally subject to complex cell-type-specific regulation involving multiple promoter usage and alternative splicing (5). A number of experimental observations strongly suggest a tumor suppressor function for tropomyosin genes (3, 7-9). The present study was undertaken to determine: 1) the expression pattern of TM isoforms in normal and malignant human breast epithelial cells; 2) the mechanism of any altered TM isoform expression in human breast cancer cells; 3) whether altered TM expression is useful as a prognostic marker; and 4) the effects of altered TM isoform expression *in vivo*. In this manner it can be determined if TMs are valid prognostic markers and/or targets for therapeutic intervention.

BODY

Methods

All experimental methods not described elsewhere (10-12) will be described in the body of this summary.

Results

Expression of RNA encoding HMW TMs is altered in HBCC. Previous analyses of TM levels in HBEC were limited. TM expression was thus examined more completely. Northern blot analysis was performed using isoform-specific DNA probes to determine TM RNA abundance in

an *in vitro* cell culture model of human breast cancer. A summary of the growth properties of the cells used in these experiments is given in Table 1.

Densitometric quantification of the results from Northern blot analysis revealed that RNA for the HMW TM isoform TM1, was moderately abundant in normal, mortal HBEC (HMEC, Clonetics Corp.) but undetectable in several HBCC lines. TM1 RNA was slightly elevated in the benign, immortalized HBEC HBL100 (approximately 4-fold) and 184A1N4 (approximately 2-fold) and also in the highly tumorigenic HBCC 184A1N4TH (approximately 4-fold) and MDA231 (approximately 2-fold), relative to normal, mortal HBEC. TM2/TM5a RNA was undetectable in normal HBEC and most HBCC, barely detectable in SKBR3 and expressed at very low levels in the benign cells 184A1N4, HBL100 and MCF10A. TM3/TM5b and slow twitch α -TM (α -TM_s) RNA were each present at similar, very low levels in normal, mortal and benign, immortalized HBEC and SKBR3 HBCC whereas they were undetectable in MCF7, T47D and MDA231 HBCC. RNA for the LMW TM isoform TM4 was moderately abundant in normal HBEC and slightly elevated in HBL100 and 184A1N4 HBEC and in 184A1N4TH HBCC (approximately 3.5-fold for each). There was no significant difference in the relative abundance of TM4 RNA in the other HBCC. RNA for TM5 was also moderately abundant in HBEC. However, TM5 RNA levels were not significantly different in any of the HBEC or HBCC examined. Overall, the TM2/5a, TM3/5b and α -TM_s RNA levels were quite low, requiring two week exposure for detection in contrast to overnight exposure required to detect TM1, TM4 and TM5 RNA using comparably labeled probes. The most striking observations were the strong expression of TM1 RNA in highly tumorigenic HBCC and its absence in the weakly tumorigenic HBCC.

Expression of HMW TM proteins is altered in HBCC. Class-specific and isoform-specific

monoclonal antibodies (mAbs) were next used to determine HMW TM protein levels in the breast cell lines. mAb TM311 (purchased from SIGMA) recognizes an amino-terminal epitope common to the HMW TMs in both muscle [α - and β -TMs, (13) and nonmuscle cells TMs 1, 2; (6). mAbs CG1 and CG β 6 are specific for TM1 and TM2/3, respectively (14, 15). Western blot analysis of HMW TM expression (10) using mAbs TM311 and CG1 revealed that normal HBEC expressed low levels of HMW TM 1 protein. The mAb CG1 confirmed the expression of TM1 identified by mAb TM311 and also revealed the presence of an additional TM1-related isoform. This isoform was also identified by mAb CG1 in MDA435 cells, which lack TM311 immunoreactivity. The amounts of TM1 in the benign cells varied with high amounts in MCF10A and normal amounts in 184A1N4. TM1 was undetectable in the weakly tumorigenic SKBR3, MCF7 and T47D HBCC and present in high amounts, relative to normal HBEC, in the highly tumorigenic MDA231 and MDA435 HBCC.

The molecular basis for the presence of the smaller TM1 isoform is unknown at present, but could be related to protein degradation or differences in epitope recognition. HMW TM levels are affected by differences in the control of their degradation (16). Moreover, CG1 recognizes an epitope on TM1, which is different from that recognized by TM311.

Western blot analysis with mAb TM311 and the TM2/3-specific mAb, CG β 6, revealed the presence of a protein species with the M_r of TM2 in HBEC and a species with the M_r of TM3 in MCF10A (distinguished upon shorter exposure of the western blot) and SKBR3 (10, 11). Both of these isoforms were present at levels consistent with their very low RNA expression (10). The differences in overall TM2 and TM3 detection (11), may reflect differences in the affinity of the 2° Ab for TM311 (IgG1 isotype) versus CG β 6 (IgM isotype). It may also reflect differences in the levels of HMW TMs in the two reduction mammaplasty isolates used as controls, HMEC (from

Clonetics; TM311) and 184 cells (17) CG β 6), differences in HMW TM degradation rates in the different breast cell lines or the extremely low levels of TM2 and TM3 expression in these cells. Nevertheless, the data clearly demonstrate that the HBEC and HBCC used in these studies express very low levels of tropomyosin isoforms 2, 3, 5a, 5b. Moreover, expression of these nonmuscle isoforms was comparable to expression of the muscle isoform slow twitch- α .

Persistent TM1 expression in HBCC is due to epigenetic mechanisms. It has been previously shown that the cytoskeletal ABP gelsolin is under epigenetic control in the same HBCC examined for TM expression (12). It was thus asked if altered TM levels in HBCC were the result of epigenetic regulation involving CpG methylation and/or histone acetylation (10). Changes in CpG methylation are associated with the altered expression of both oncogenes and tumor suppressor genes (18). Methylation may affect transcription factor access to a gene either by direct steric interference of binding or by altering chromatin structure (19). The acetylation state of histones is another important regulator of gene expression (20, 21). The effects of histone deacetylase (HDAC) inhibitors, such as butyrate and Trichostatin A (TSA), on the growth and differentiation of several different types of cancer cells suggest that alterations in histone acetylation contribute to the transformed (22, 23). Inhibition of HDACs by specific inhibitors leads to increased acetylation of (23), and often results in enhanced transcription, presumably because hyperacetylation of histones increases accessibility of nucleosomal DNA to transcriptional regulatory proteins reviewed (21). MCF7, T47D and MDA231 HBCC were therefore treated with the demethylating agent 5-azacytidine (5aza), the HDAC inhibitor TSA or both, and TM1 RNA and protein levels were examined in the treated and untreated cells.

Northern blot analysis was performed on RNA from untreated, control 6aza-(inactive for

demethylation), 5aza-, TSA- or TSA plus 5aza-treated MDA231, MCF7 or T47D breast cancer cells. TM1 protein levels in MCF7 or T47D cells were unaffected by any of the treatments (data not shown). Fig. 1A shows the results obtained with MDA231 cell RNA. Both 5aza and TSA down-regulate TM1 RNA expression (>65% and >99% maximum reductions, respectively) in MDA231 cells. The effects of TSA plus 5aza treatment were similar to TSA alone. TM1 RNA abundance in MCF7 and T47D cells was unaffected by any of the treatments (data not shown). Fig. 1B shows the results of Western blot analysis of protein extracts derived from untreated, control 6aza, 5aza-, TSA-, or TSA plus 5aza-treated MDA231 cells using mAb TM311. This analysis revealed down-regulation of TM1 protein by >27%, >60% and >80% after treatment with 5aza, TSA and TSA plus 5aza, respectively.

Expression of LMW TM isoforms is also altered in HBCC. Fig. 2 shows the results from Western blot analysis was performed on extracts prepared from HBEC and HBCC using mAb TM228 (SIGMA; which reacts with a 36kD TM isoform in chicken gizzard extracts), mAb LC24, which is specific for the LMW TM isoform, TM4, and mAb LC1, which is specific for the LMW TM isoform, TM5 (14, 15). mAb TM228 detected a LMW TM isoform in mouse fibroblasts, normal, mortal 184 HBEC and the benign, immortalized MCF10A HBEC. The M_r of this isoform differs from that detected in chicken gizzard extracts by comparison to TM4 or TM5 (with reported M_r s of 30-33kD; (2, 14), so it has been arbitrarily term it TM33 based on its observed mobility. There is no detectable reactivity with mAb TM228 in extracts from SKBR3, MCF7 and T47D whereas it detects elevated levels of the LMW TM33 isoform in MDA231 and MDA435 extracts, relative to normal HBEC.

The TM4-specific mAb LC24 reveals that 3T3-L1 fibroblasts lack TM4 immunoreactivity.

Surprisingly, the antibody detected two TM4-related isoforms. In normal HBEC and each of the benign HBEC there is a strong major band and a weak minor band (only visible upon longer exposure in normal and 184A1N4 HBEC). Elevated TM4 protein in 184A1N4 cell extracts correlates with higher TM4 RNA levels in those cells. MDA231 HBCC express both isoforms (present at a different ratio than in HBEC) and MDA435 HBCC express only the upper isoform. As was seen for TM1 isoforms, HBCC also show dramatic differences in TM4 isoform composition. SKBR3, MCF7 and T47D HBCC lack detectable TM4 protein (the T47D lane signal is spillover of the MDA435 extract) whereas expression is strong in MDA231 and MDA435 HBCC. This contrasts with TM4 RNA expression, which does not show any significant reduction in the breast cancer cell lines relative to normal HBEC.

The TM5-specific mAb LC1 reacts with a HMW TM isoform in 3T3-L1 fibroblasts while in most of the breast cell lines there is a single LMW immunoreactive species (MCF10A also has a faint upper band). In contrast to TM5 RNA levels, which show little difference between the breast cell lines, TM5 protein expression is altered in the HBCC. Thus, SKBR3, MCF7 and T47D cells have much reduced expression while MDA231 and MDA435 cells have elevated expression, relative to normal HBEC. A second TM5-specific mAb, CG3, (24) has confirmed the TM5 expression pattern identified by mAb LC1 (not shown).

Treatment with proteasome or kinase inhibitors up-regulates LMW TM abundance in HBCC. TM protein levels in transformed fibroblasts are affected by treatment with the cysteine protease inhibitor, Calpain Inhibitor I (also known as LLnL)(16). Additionally, LMW TM isoforms are not turned over as rapidly as are HMW TM isoforms (16). Moreover, comparison of TM4 and TM5 RNA and protein levels suggests that LMW TM expression in HBCC is regulated post-

transcriptionally at the level of protein abundance. It was thus asked if LMW TM expression in the low TM abundance HBCC, MCF7 and T47D, could be upregulated by treatment with proteasome inhibitors. MCF7 and T47D HBCC were treated with Calpain inhibitor I (CI), which will inhibit activity of free 19S and 20S subunits as well as the fully assembled 26S proteasome, and with MG132, which is a specific inhibitor of the 26S proteasome (25), then examined TM4 and TM5 levels by Western blot analysis with the isoform-specific mAbs LC24 and LC1 or CG3. As shown in Fig. 3A, treatment of MCF7 HBCC with either inhibitor upregulated TM4 and TM5 expression relative to untreated or vehicle treated cells. CI treatment had a greater effect on TM4 and TM5 levels than did MG132. Treatment with both inhibitors upregulated LMW TM levels in T47D HBCC to a lesser extent (data not shown). TM5 levels in benign, immortalized 184A1N4 HBEC were up-regulated only after treatment with CI while TM4 levels appeared to be unaffected by either treatment (data not shown). TM1 expression also did not appear to be affected in either of the HBCC after treatment with the proteasome inhibitors (data not shown).

The HBCC were also treated with the protein kinase inhibitors H89 (1 μ M), chelerythrine chloride (Che-Cl, 3 μ M) and rapamycin (30nM) to determine if TM protein abundance in HBCC was regulated by signal transduction pathways which use the targets of these molecules. Results of these experiments demonstrated upregulation of TM4 in MCF7 HBCC after treatment with each kinase inhibitor (Fig. 3B). Similarly, TM4 and TM5 were also upregulated after treatment of SKBR3 and 184A1N4TH HBCC, respectively, with each kinase inhibitor (not shown). TM1 abundance was increased in SKBR3 and 184A1N4TH HBCC after treatment with H89 and Che-Cl, respectively (Fig. 4). The only change noted in the benign HBEC was a slight upregulation of TM5 in the HBL100 line (Fig.4).

Expression of HMW TMs is altered in human breast cancer tissues. Preliminary experiment using indirect immunofluorescence (IF) with mAb TM311, had revealed moderate to strong cytoplasmic staining in normal HBEC and benign HBEC lines while most HBCC lines showed reduced staining (data not shown). Immunohistochemistry (IHC) was then employed using mAb TM311 on sections from frozen and paraffin embedded normal breast and breast tumors (10, 11). These analyses revealed intense HMW TM staining in myoepithelial cells and moderate staining in luminal cells of ducts and acini (see Fig. 5, panel A for an additional example). TM311 stained smooth muscle within the vasculature of the breast tissue strongly and this provided a good internal positive control for antibody reactivity (Fig. 5 panels A-D). Controls for normal HMW TM expression consisted of sections of normal breast tissue stained in the same run as the tumor sections and residual normal components within a section. In the normal breast tissue stained with mAb TM311, the staining intensity of the myoepithelial cells was labeled as 3+ and the intensity of luminal cell staining was labeled as 2+. Three HMW TM staining patterns were identified in the tumor sections using mAb TM311. There were tumors with negative or reduced TM staining (0/1+), tumors with normal staining (2+) and tumors with increased TM staining (3+). Fig. 5 gives examples of each staining pattern we observed. Overall, reduced HMW TM immunoreactivity occurs in approximately 30% of the tumors examined (n=62). Thus there are a number of tumor sections (n=46) which are positive with mAb TM311, suggesting that normal HMW TM expression is retained in these tumors. However, in a large percentage of the positive cases (37%), a proportion (avg.=34.5%) of the positive tumor cells have a stronger immunoreactivity than the corresponding normal epithelium control. This allowed us to characterize HMW TM expression in the tumors as either reduced, normal or overexpressed.

Examination of the tumor database revealed several significant correlations. Reduced HMW TM expression correlated with age at diagnosis (Fisher's exact test, $p=0.0279$; Table 2) and negative progesterone receptor (PR) status (Fisher's exact test, $p=0.0153$; Table 3). In this respect, there were also two interesting trends noted. There were not quite significant associations between HMW TM overexpression and both negative PR and negative estrogen receptor (ER) status (Fisher's exact test, $p=0.0827$ and $p=0.0518$, respectively, data not shown). Fig. 6 shows Kaplan-Meier survival curves for patients in the different TM expression groups. The mean follow-up time for this cohort was 3.92 (± 1.5) years. Life table analysis (26) revealed a significant difference in survival between patients with reduced HMW TM expression and those with normal TM expression (chi-square = 4.505, $p = 0.0338$; Fig. 6, top and bottom curves, respectively). The differences in survival between HMW TM overexpressors and patients with either reduced or normal TM expression were not significant.

The significant association of TM expression levels with PR status in the tumors prompted an examination of survival in the different PR subsets. The results of that analysis are shown in Fig. 7. Although the numbers are small, the overall survival in the PR+ subset is consistent with that found in the total population. The patients whose tumors expressed normal levels of HMW TM exhibited much poorer survival than those whose tumors showed reduced or overexpressed HMW TM (Fig. 7, left panel, compare with Fig. 6). A surprising finding in the PR- subset was that those patients with tumors that overexpressed HMW TM had a similarly poor survival to those patients in the PR+ subset whose tumors had normal HMW TM levels (Fig 7, right panel, compare with left panel).

Discussion

TM expression in normal HBEC. Non-muscle cells typically express 5-8 isoforms of TM (5, 27). The functional basis for this diversity is not clear but is generally thought to be related to the expanded function of microfilaments in non-muscle cells (6, 27). The HBEC examined in this study appear to have a different TM expression profile than fibroblasts, but the expanded expression of TMs in both cell types includes the presence of several LMW TM isoforms (25). The identification of both HMW and LMW TM proteins in normal HBEC contrasts with the TM profile of human intestinal epithelial cells. Normal epithelial cells isolated from the jejunum and colon exclusively express LMW TMs (14). This contrast could be related to the structural and functional differences between these two tissues. Although similarly polarized, the two epithelial components differ from each other in the mode and directionality through which they transport substances. The intestinal epithelium uses both paracellular and transcellular routes for bidirectional transport of water and solutes whereas the mammary epithelium uses a predominantly transcellular mode, primarily for the export of substances. Moreover, this activity is maximal only after the functional differentiation induced by pregnancy and lactation. Additionally, the number and types of intercellular connections as well as cell-matrix attachments within these compartments are different cell-cell and cell-stromal interactions differ between the two compartments (28). It is therefore not surprising that the TM isoform composition of their actin cytoskeletons should differ.

TM expression in benign HBEC and malignant HBCC. Prior studies examining HMW TM expression in HBCC lines and human breast tumor cells used 2-DE to identify the TM isoforms, based on comparison to a reference profile obtained using proteins extracted from fibroblasts (2, 3). The present studies demonstrate that normal and benign, immortalized HBEC differ from fibroblasts in their overall TM expression profile, suggesting that fibroblast TM expression may not be an ideal

control for comparison purposes (Fig. 4 and data not shown). In general, it was found that benign, immortalized HBEC showed either no change or a slight upregulation of TM isoform expression compared to normal, mortal HBEC. In the HBCC, TM1 protein abundance was consistent with that for TM1 RNA, suggesting that changes in TM1 expression were due to altered regulation of RNA levels. This was most clearly evident in MDA231 HBCC where persistent (slightly elevated) TM1 RNA was due to epigenetic regulation. TM4 and TM5 levels in HBCC were regulated post-transcriptionally at the level of protein abundance. Previous studies have demonstrated differences in the rates of synthesis and/or turnover of HMW versus LMW TM proteins (16, 29). Some studies have implicated proteasome-mediated degradation in the down-regulation of HMW TM observed during transformation of fibroblasts (16). No changes were observed in HMW TM expression after treatment with proteasome inhibitors. On the contrary, this study has shown upregulation of LMW TM isoform levels in MCF7, T47D and MDA231 HBCC but not in benign 184A1N4 HBEC after treatment with the proteasome inhibitors MG132 and calpain inhibitor I. This suggests that different TM levels in some HBCC may result from altered degradation rates. The greater induction of TM4 and TM5 by calpain inhibitor I suggests that changes in non-proteasomal degradation of LMW TMs also occur in HBCC. Differential effects were also observed on TM expression in HBEC versus HBCC after treatment with three different kinase inhibitors, suggesting altered cell signaling in HBCC, affecting TM expression. Altered TM isoform composition in HBCC therefore resulted from changes in both synthesis and degradation of both classes of TMs, implying that dysregulation of TM is an important step in the progression to malignancy in breast epithelial cells. The net result of this dysregulation being a global up- or down-regulation of TM protein levels.

Remarkably, the pattern of changes in TM isoform levels in HBCC were inversely

proportional to their *in vivo* growth potential (see Table 1). Non-tumorigenic and weakly tumorigenic, non-metastatic HBCC exhibited down-regulation of both HMW (TMs 1 and 2) and LMW (TMs 4, 5 and 33) TM expression. Conversely, highly tumorigenic, metastatic HBCC exhibited upregulation of most of these TM isoforms. These data suggest that specific TM expression profiles are associated with the development and/or maintenance of specific phenotypes in HBCC. Elevation of TM expression in benign, immortalized HBEC suggests that, for highly invasive or metastatic phenotypes, this may occur earlier during tumor progression.

Elevated TM expression has been associated with invasive potential. Elevated TM1 levels have been noted in the cells from node positive tumors (relative to node negative tumors) and elevated TM5 has been noted in highly metastatic mouse melanoma cells relative to low metastatic melanoma cells (3, 30). In the latter study, transfection of an inducible antisense TM5 cDNA resulted in reduced TM5 protein levels and dramatically decreased motility of the transfected cells under induced conditions. This suggested that TM5 is important for the regulation of motility in malignant melanoma cells, which, in turn, is necessary for invasion and metastasis.

A change in the pattern of TM expression will produce altered TM isoform ratios. TM isoform ratios determine TM dimer formation and thus actin binding potential. For example, HMW TM homodimers (i.e. α/α or β/β) are predicted to have a reduced head-to-tail overlap relative to α/β heterodimers which may be responsible for the *in vitro* observation of reduced F-actin binding for the (27, 31). TM4 has a reduced affinity for F-actin compared to other LMW TMs. TM5, however has an F-actin affinity similar to that of muscle TMs. Moreover, LMW TMs are capable of homo- and heterodimerization and the dimers will interact in a head-to-tail fashion, which will affect actin binding properties (6). Some LMW TM isoforms do not protect the actin filament from severing

proteins as well as HMW TMs, nor do they interact as strongly with other actin-binding proteins such as caldesmon (32), suggesting that cells over-expressing multiple LMW TMs will have a less stable cytoskeleton. The observed upregulation of three LMW TM isoforms in the highly tumorigenic, metastatic HBCC implies a profoundly altered HMW:LMW TM isoform ratio. These findings suggest the hypothesis that the TM expression profile in invasive or metastatic cells reflects the need for a more fluid cytoskeleton, similar to that observed for normal, highly motile cell populations such as macrophages, in which HMW TM expression is (33). The differences observed in this study might thus be important for determining the phenotype of HBCC. This hypothesis is currently being tested in the context of the normal breast epithelium environment.

TM expression in human breast tumors. The IHC data clearly demonstrate HMW TM expression in the luminal cells of normal ducts and acini, which had not been previously assessed. Additionally, luminal cells of normal ducts and acini also express the LMW TM5 isoform (data not shown). This is important because luminal cells are thought to be the progenitor cells of breast cancers (34). Importantly, a pattern of altered HMW TM isoform expression similar to that observed in the cell culture model, was observed in breast tumors. LMW TM isoform expression is currently being examined in the same tumors and preliminary results suggest that TM5 expression will be altered in a similar fashion (data not shown).

Examination of the clinical-pathological parameters for these patients revealed associations between HMW TM levels and age at diagnosis, ER status and PR status. The association between increased HMW TM expression and ER positivity reflects that which was found in the cell culture model. The significant correlation of reduced HMW TM levels and PR negativity, however, is the opposite of that seen in the cell culture model. The most important relationship identified thus far

is the significant correlation of normal TM expression levels with reduced overall survival. In this respect, the differences in survival noted in the PR+ versus PR- patient subsets suggest that hormone receptor status and altered TM expression may be related. There was no significant relationship between HMW TM expression and any other clinical-pathological parameter. This may have something to do with the size of the cohort examined so far. We are currently accruing more samples for analysis. Samples archived prior to 1993 were fixed in Lilly's fixative, which may affect either the antigen-antibody interaction or the efficiency of antigen retrieval (Mielnicki and Asch unpublished data). For this reason, most of the tumor specimens examined thus far have been fixed in formalin. A few of the specimens in this cohort were Lilly-fixed specimens that were reactive with mAb TM311. Thus, sections from older Lilly-fixed tissues are currently testing for their reactivity with anti-TM mAbs utilized in this study.

The significance of these observations is unclear; however, elevation of HMW and LMW TM expression has biological consequences in the cells in which it occurs (8, 30, 35). This suggests that normal TM expression patterns are essential for proper cell function. Alterations of normal TM function do result in diseased states of both muscle and nonmuscle tissues (7, 36). The data from the present studies suggest that this may also be the case for human breast cancer.

Recommendations related to the statement of work (sow):

With respect to Technical Objective 1, task 1, relating to the preparation of histological specimens and DNA/RNA probe constructs is complete. The titration of antisera for IHC analysis only, has been hampered by the loss of reactivity during storage at -20C of the CG1 antiserum. However, the TM5 specific antiserum, CG3 is still potent and is being used for IHC analysis on tumor section. The supply of antisera has become a limiting factor in the completion of some of the tasks. CG1,

LC24, and LC1 supplies are depleted and more must be obtained from Dr. J. Lin. Antisera obtained from Dr. D Helfman, has not been reliable, and either more conditions must be tried or more must be obtained. Task 2 is also completed. Completion of task 3 has been hampered by the inability to obtain the required sections in a timely manner from the pathology department at RPCI. The sections described above were obtained only recently. However, analysis of HMW TM RNA in the tumors is not necessary as per the correlation of TM1 RNA and protein expression in the cell lines and the very low levels of TMs 2, 3, 5a and 5b in the cultured cells. This will be confirmed in HMW TM positive tumor and normal breast sections with the TM2/3specific antiserum. This will allow the unequivocal assignment of TM1 as the antigen reactive with mAb TM311, obviating the need to obtain more of mAb CG1. Task 4 has been completed with for all tumors examined thus far.

With respect to Technical Objective 2, experiments designed to address tasks 1 and 2 have determined which regulatory mechanisms are affecting the expression of both HMW and LMW TM expression in the HBCC. Task 3 is no longer relevant due to a lack of experimental evidence for alterations at TM loci in human cancers other than the TRK translocations identified in thyroid carcinomas (e.g. loss of heterozygosity). Work has also begun on preparing the constructs described in task 4. A tetracycline regulated TM1 expression vector has been prepared and transfected into MCF7 Tet-off cells (Clontech). The transfectants have been put into selection and several clones as well as a mixed stable population have been derived from them. These lines will shortly be characterized for inducibility of TM1 and in the assays required for Technical Objective 3. Work is also currently proceeding on the construction of the Tet-regulated TM4 and TM5 constructs described in technical objective 2.

Regarding Technical Objective 3, task 1 is well underway, as evidenced in the previous

section. In addition, as indicated in the Reportable outcomes section, stable pTet-off (tet-regulated transactivator construct) transfection of six HBEC and HBCC has resulted given rise to several lines (as well as mixed stable pools) which are necessary for the experiments described in Technical Objective 3. Thus, work on task 1 is proceeding successfully and, although not complete, work on this and the other tasks in this objective should move forward without delay.

KEY RESEARCH ACCOMPLISHMENTS

- ☞ Tropomyosin isoforms (TMs) 1, 2, 4, 5 and 33 have altered expression in human breast cancer cells (HBCC) relative to normal and immortalized human breast epithelial cells.
- ☞ TMs 1 and 2 (high molecular weight, HMW) have altered regulation of RNA abundance in HBCC.
- ☞ Persistent TM1 expression in MDA231 HBCC is due to epigenetic regulation.
- ☞ Reduced levels of TMs 4 and 5 (low molecular weight, LMW) in HBCC is through regulation of protein abundance via increased proteasomal and calpain mediated degradation.
- ☞ Altering signal transduction in HBCC, but not HBEC, affects TM expression.
- ☞ HMW TM expression is reduced in 25.8% of breast tumors examined.
- ☞ HMW TM expression is elevated in 25.8% of breast tumors examined.
- ☞ HMW TM expression is normal in 48.4% of breast tumors examined.
- ☞ Reduced TM expression in human breast tumors is correlated with premenopausal status.
- ☞ Reduced TM expression in human breast tumors is correlated with negative progesterone receptor status.
- ☞ TM overexpression in human breast tumors is associated with negative progesterone receptor status.

- ☞ TM overexpression in human breast tumors is also associated with positive estrogen receptor status.
- ☞ Reduced TM expression in human breast tumors is correlated with increased survival whereas normal TM expression is correlated with poor survival.

REPORTABLE OUTCOMES

1. Mielnicki LM, Ying AM, Head KL, Asch HL, Asch BB. Epigenetic regulation of gelsolin expression in human breast cancer cells. *Exp Cell Res* 1999; 249:161-76.
2. Mielnicki LM, Heiberger KL, Ying AM, Asch HL, Asch BB. Aberrant regulation of tropomyosin isoform expression in human breast cancer cells. *Proc AACR* 1999; 40:434-5, abstract.
3. Mielnicki LM, Ying AM, Heiberger KL, McCabe MM, Asch HL, Asch BB. Deregulation of tropomyosin expression in human breast cancer. *Proc DOD BCRP EOH* 2000; 1:124, abstract.
4. Mielnicki LM, Ying AM, Heiberger KL, McCabe MM, Asch HL, Asch BB. Aberrant regulation of tropomyosin isoform expression in human breast cancer. 2000. Manuscript in preparation.
5. Development of HBEC and HBCC cell lines stably expressing the pTet-Off cDNA (Clontech); pTet-Off lines include derivatives of 184A1N4, MCF10A, HBL100, MCF7, T47D and MDA231.
6. Development of MCF7 derivatives expressing a tetracycline inducible TM1 expression construct.
7. Grant applied for from NCI/NIH (RO1 application) to expand on the present findings; direct costs \$175,000/yr for 4 yrs.
8. Applications pending for faculty positions at SUNYAB, Dept. of Biochem. and RPCIC, Dept. of Cancer Genetics.

CONCLUSIONS

Prior to the present work, expression of individual TM isoforms in normal and malignant breast epithelial cells *in situ* had not been assessed. In fact, only MDA231 and MCF7 HBCC had been examined for TM RNA expression (1, 57). We have demonstrated altered TM expression *in situ* in invasive breast tumors. We have confirmed the earlier TM RNA findings and provided a more complete analysis of TM RNA and protein expression in HBEC of differing malignant potentials (i.e. benign Vs carcinoma, weakly Vs highly tumorigenic). Our data show alterations in LMW as well as HMW TM expression in HBCC. This is the first demonstration of aberrant LMW TM isoform expression in human breast cancer. These data suggest that tropomyosin isoform expression is a common target for alteration during breast epithelial cell transformation. The association of TM expression levels with specific outcomes in human breast cancer patients suggests that TM isoform composition can affect tumor cell behavior *in vivo*, as it does *in vitro* in tissue culture cell systems. This, in turn, suggests that TM isoform composition may have some prognostic value. The ability to manipulate TM isoform levels, and thus TM expression profiles, in HBCC in culture suggests that TMs represent potential therapeutic targets.

These initial promising data require follow-up to address the specific role of each TM isoform in invasion and metastasis. These experiments, utilizing inducible TM constructs and 3-D culture systems will soon be underway. Tissue specific, transgenic approaches to examine the role of TMs in normal mammary development and mammary tumorigenesis are also being planned. In addition, IHC analysis of human breast tumors will continue, in order to include as many samples as possible and thus provide more statistical power to the findings. The analyses will be expanded to include all relevant TM isoforms, as indicated by the present data. This aspect is currently moving

forward.

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Table 1. Characteristics of HBEC Used in the Present Studies.

Cell line	Source	<i>in vitro</i> growth properties		<i>in vivo</i> growth properties		
		soft agar growth	Invasive	tumors	metastatic	references
HMEC	Reduction Mammoplasty	No	No	No	–	(37)
184	Reduction Mammoplasty	No	No	No	–	(17)
184A1N4	184 cells ^a	No	No	No	–	(17)
MCF10A	MCF10 cells ^b	No	No	No	–	(38)
HBL100	Human milk	Yes ^c (moderate)	ND	Yes ^d	–	(39)
SKBR3	PE ^e	No	Yes(low)	No	–	(40)
MCF7	PE	Yes	Yes(low)	Yes	No	(38, 40)
T47D	PE	Yes	Yes(low)	Yes	No	(40, 41)
MDA231	PE	Yes	Yes(high)	Yes	Yes	(40, 42)
MDA435	PE	Yes	Yes (medium)	Yes	Yes	(40, 43)
184 TH	184 A1N4 cells ^f	Yes	Yes	Yes	Yes	(17, 44)

^aBenzo(a)pyrene immortalized 184 cells; ^bSpontaneously immortalized from normal, mortal HBEC; ^clate passage cells (P66 or greater); ^dlater passage cells (P70 or greater); ^ePE, pleural effusion; ^ftransfected with SV40 T antigen and activated c-Ha-ras

Table 2. Analysis of HMW TM expression versus age at diagnosis.

	Normal TM	Reduced TM
age < 46yrs.	5	8
age > 45yrs.	20	5

Comparisons were made using Fisher's exact test, $p=0.0279$, Odds ratio=0.1563, 95%CI [0.035 to 0.691].

Table 3. Analysis of HMW TM expression versus PR status.

	Normal TM	Reduced TM
PR+	14	3
PR-	7	11

Comparisons were made using Fisher's exact test, $p=0.0153$, Odds ratio=7.333, 95%CI [1.531 to 35.128].

Table 4. Associations between patient survival, clinicopathologic parameters, and breast cancer HMW TM expression.

Parameter	Category	No. Patients		P ^b	Odds Ratio (95% CI) ^c
		Dead ^a	Alive		
Pathologic stage	I + II	9	59		
	III + IV	7	7	0.005**	6.56 [1.86 to 23.1]
Pos. lymph nodes	<3	4	41		
	>2	8	26	0.048*	4.11 [1.1 to 15.5]
Estrogen Receptor	Negative	9	20		
	Positive	6	48	0.154	2.42 [0.77 to 7.6]
HMW TM	Reduced	1	14		
	Normal	18	10	0.034*	6.77
	Overexpressed	10	3		[1.1 to 12.4]

^a Deaths due to breast cancer. Deaths due to other causes (2 cases) were deleted. ^b P values were calculated by Fisher's Exact test except for HMW TM (log rank test). Additional associations between pathologic/clinical stages and survival were also significant, as follows: pathologic stages I vs. II vs. (III + IV), N = 82 cases, χ^2 test, p = 0.007; TNM stages (T0 + T1) vs. (T2) vs. (T3 + T4), N = 80 cases, χ^2 test, p = 0.005; TNM stages (T0 + T1 + T2) vs. (T3 + T4), N = 80 cases, Fisher's Exact test, p = 0.005. Other associations between survival and clinicopathologic parameters, all not significant, are as follows (parameter, P value): chemotherapy (yes vs. no); 0.18; necrosis, 0.31; Tamoxifen therapy (yes vs. no), 0.41; positive lymph nodes (0 vs. >1), 0.53; radiation therapy (yes vs. no), 0.58; nuclear grade, 0.68; size, (cutoff 1.5 cm.), 0.72; S-phase, 0.78; ploidy, 0.79; size (cutoff 2 cm.), 1.00; age, 1.00; progesterone receptor, 1.00; surgery (lumpectomy vs. mastectomy) 1.00. ^c CI is the 95% confidence interval for the Odds ratio except for HMW TM (hazard ratio).

*statistically significant values.

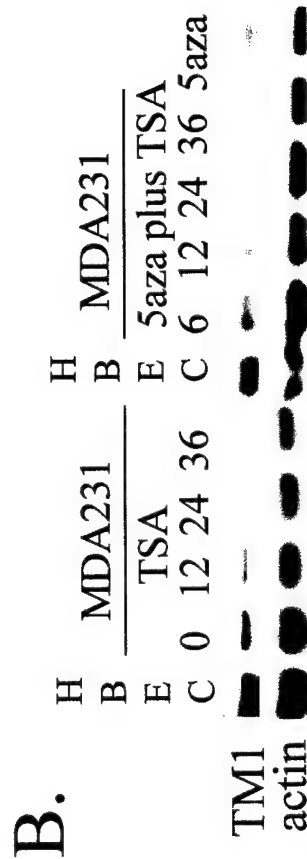
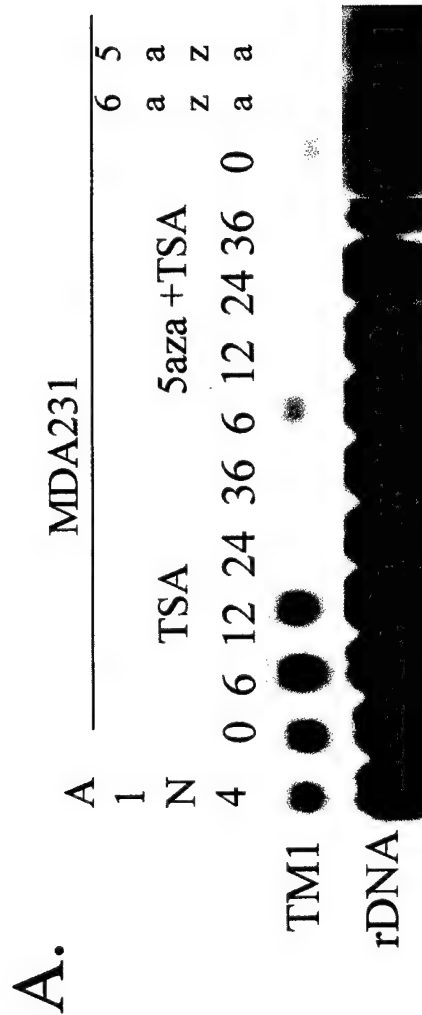


FIGURE 1. Epigenetic regulation of TM1 expression in MDA231 cells. A, Northern blot analysis of MDA231 cell RNA after treatment with the indicated inhibitors. The TM1-specific DNA probe, methods and controls were the same as in Fig. 1. B, Western blot analysis of TM1 expression in MDA231 cells after treatment with indicated inhibitors. TM1 protein was detected with mAb TM311. Actin signals are shown below. Protein loading and transfer were monitored as in (refs 1 and 2). Signals were quantified as in (refs 1 and 2) and values given in the text are based on comparison of the ratios of TM to control signals (rRNA or actin). The results shown are representative of two independent experiments.

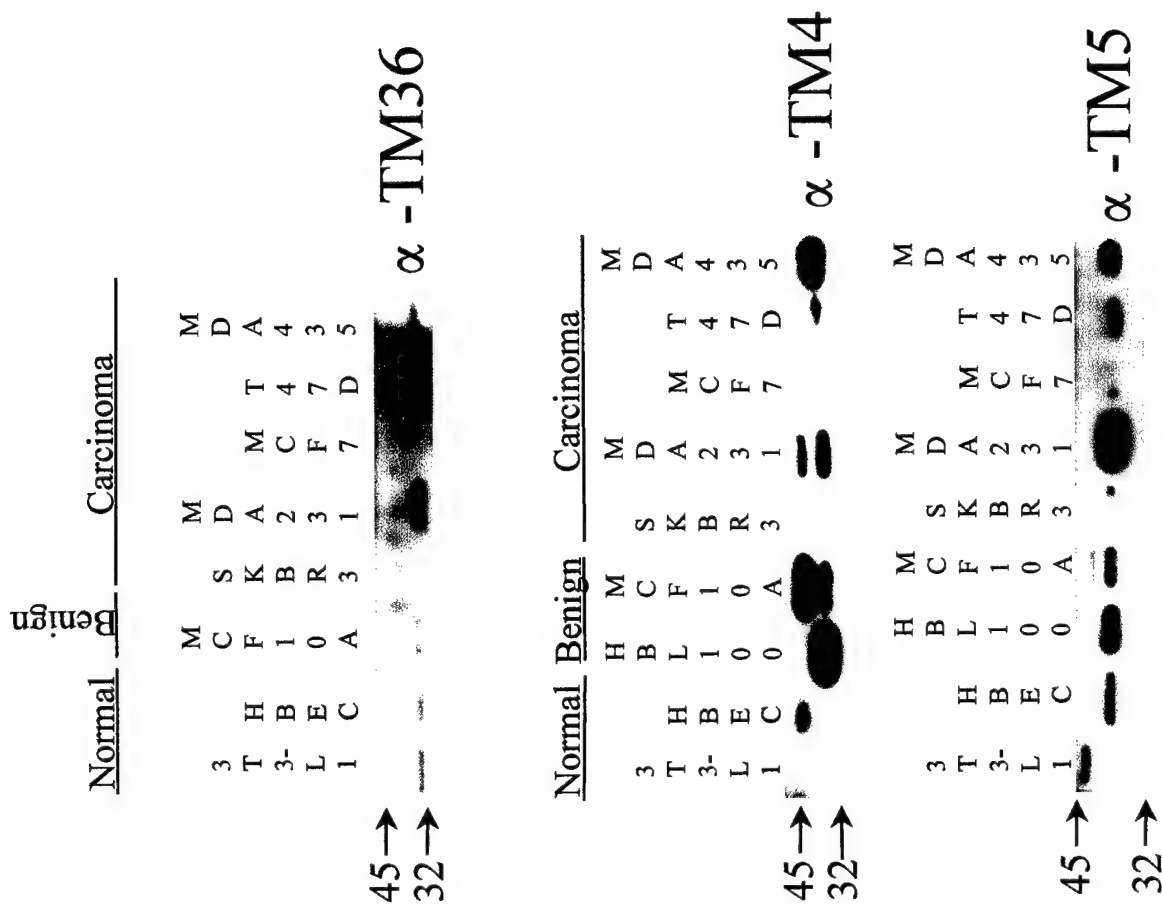
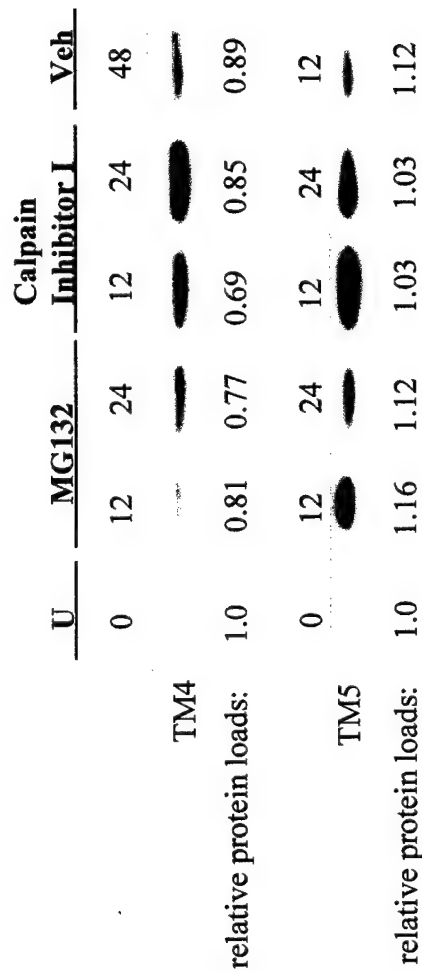


FIGURE 2. Western blot analysis of LMW TM protein expression in HBEC and HBCC. The positions of the molecular weight standards are shown on the left (arrows). Protein loading and transfer were monitored, and signals were quantified as in Fig. 1. Comparisons given in the text are based on normalization to total protein loaded per lane. The results shown are representative of two independent experiments.

A.



B.

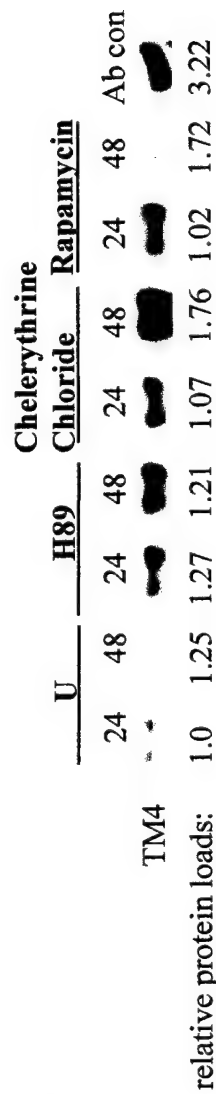


FIGURE 3. Western blot analysis of LMW TM protein in MCF7 HBCC treated with kinase or proteasome inhibitors. A, MCF7 cells were treated with either MG132 (MG), the Calpain inhibitor LLnL (CI) or vehicle (DMSO control, C). The duration of treatment (in hours) is given above each lane. B, MCF7 cells were treated with the protein kinase inhibitors H89 (1uM), chelerythrine chloride (Che-Cl, 3uM) and rapamycin (30nM) for the times indicated (in hours) above the lanes. TM4 and 5 were detected with the isoform-specific mAbs described in Materials & Methods (LC24, LC1 and CG3). Relative protein loads are shown below each signal. Protein loading and transfer were monitored and signals were quantified as in Fig. 1. Values given in the text are based on comparison of the ratios of TM to actin signals (TM4) or of TM to total protein loaded per lane (TM 5). Ab con=MDA231 extract, which was reactive with all antisera and thus served as a control for Ab reactivity. The results shown are representative of two independent experiments.

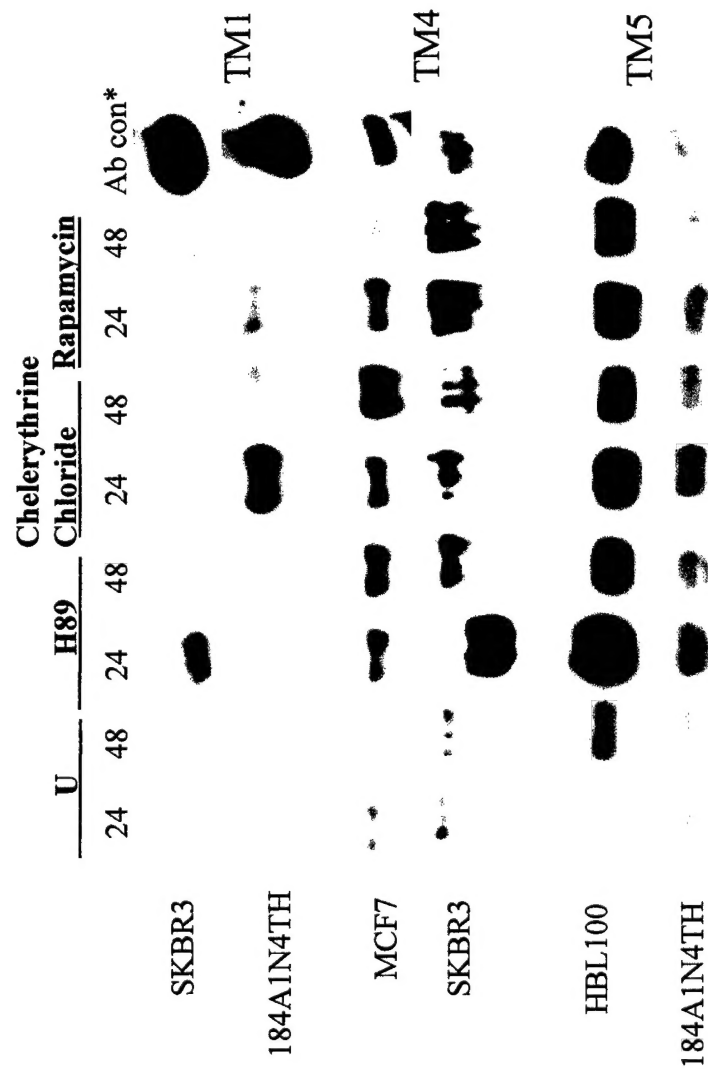


FIGURE 4. Western blot analysis of TM expression in HBL100 and HBL100 treated with protein kinase inhibitors. Protein loading and transfer were monitored, and signals were quantified as in Fig. 1. Treatment conditions and times are shown above the lanes. Cell lines are indicated on the left and TM isoforms are indicated on the right. *For SKBR3, TM4 and 184A1N4TH, TM5 signals, the control is 48h of vehicle (DMSO) treatment. Comparisons given in the text are based on normalization to total protein loaded per lane. Protein loads differed by less than twofold for all cell lines except for the HBL100, 24h H89 treatment lane, which was approximately 5-fold greater. The results shown are representative of two independent experiments.

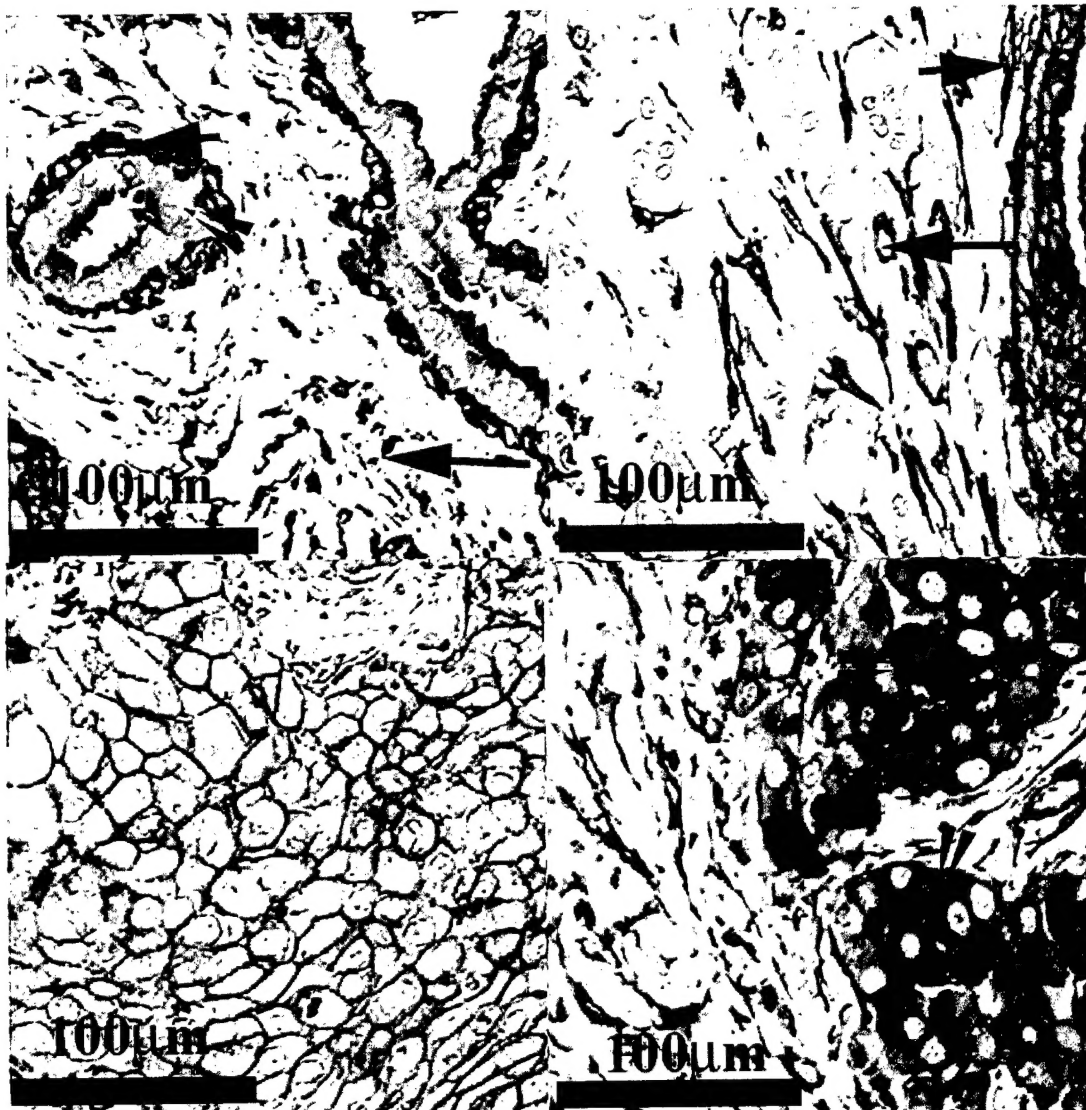


Figure 5. Immunohistochemistry analysis of HMW TM expression in paraffin embedded tumors using mAb TM311. Panel A) Normal breast, panel B) Negative tumor, panel C) HMW TM positive tumor and panel D) Tumor over-expressing HMW TM. Sections were stained as described in the text. Slides were examined and photographed on an Olympus Model BX40 microscope using a Hitachi Model KP-D50 color digital camera. A scale bar is shown in the lower left corner of each panel. Note the difference in staining intensity between the luminal (open arrow) and myoepithelial (filled arrow) cells in panel A. Note also the positive stromal components (fibroblasts and smooth muscle of vasculature, long arrows; panels A and B, respectively), residual normal ducts (short arrow, panel B) and the TM over-expressing cells (panel D, open arrows).

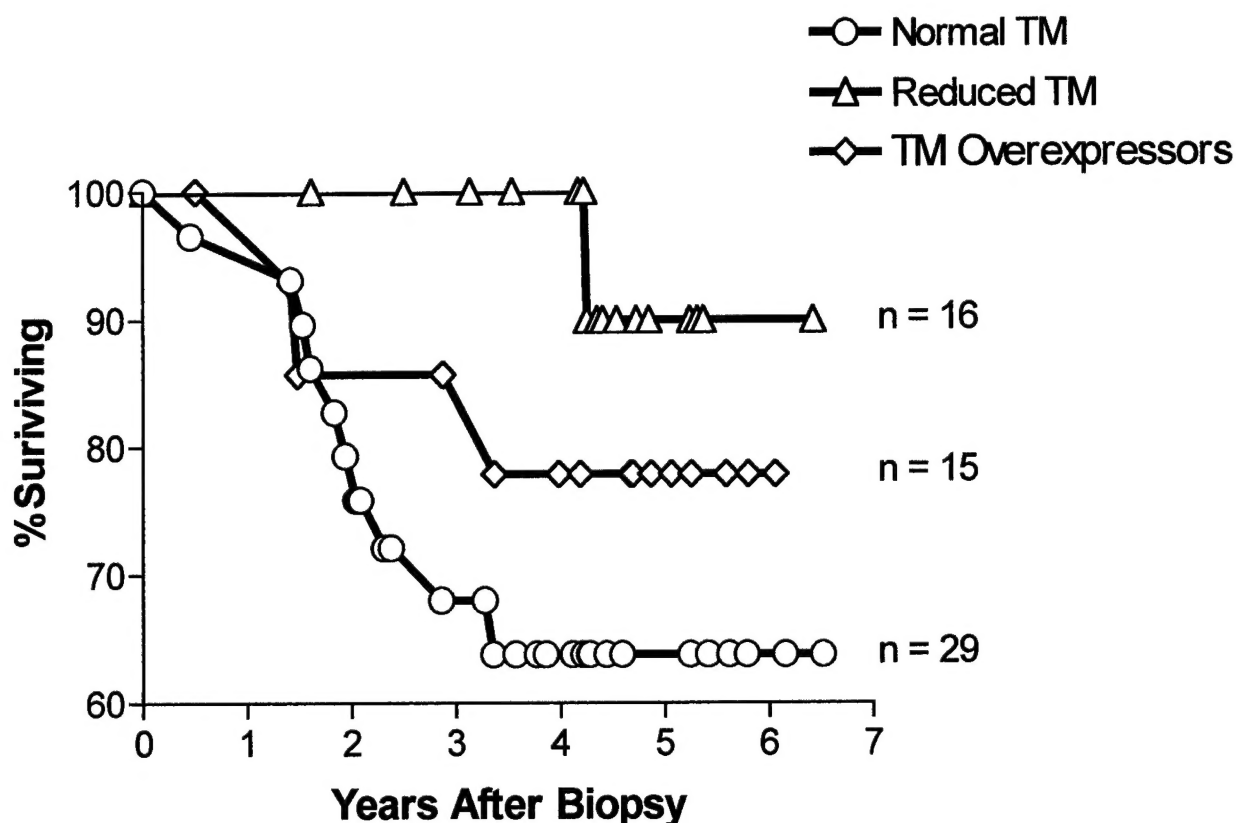


FIGURE 6. Survival analysis of breast cancer patients with different levels of tumor HMW TM expression. A Kaplan-Meier plot of survival using follow-up data from patients whose tumors had the indicated level of HMW TM expression. Life Table analysis revealed a significant difference between the survival curve of reduced HMW TM expressors and that of normal HMW TM expressors, with a p-value of 0.0338. The p-value across all three curves was not quite significant at 0.0923. The p-values from a comparison of HMW TM overexpressors with either reduced HMW TM expressors or normal HMW TM expressors were 0.2351 and 0.3672, respectively. The mean follow-up times among the groups were not significantly different ($p=0.2252$ One way ANOVA, Kruskal-Wallis analysis with Dunn's post-test).

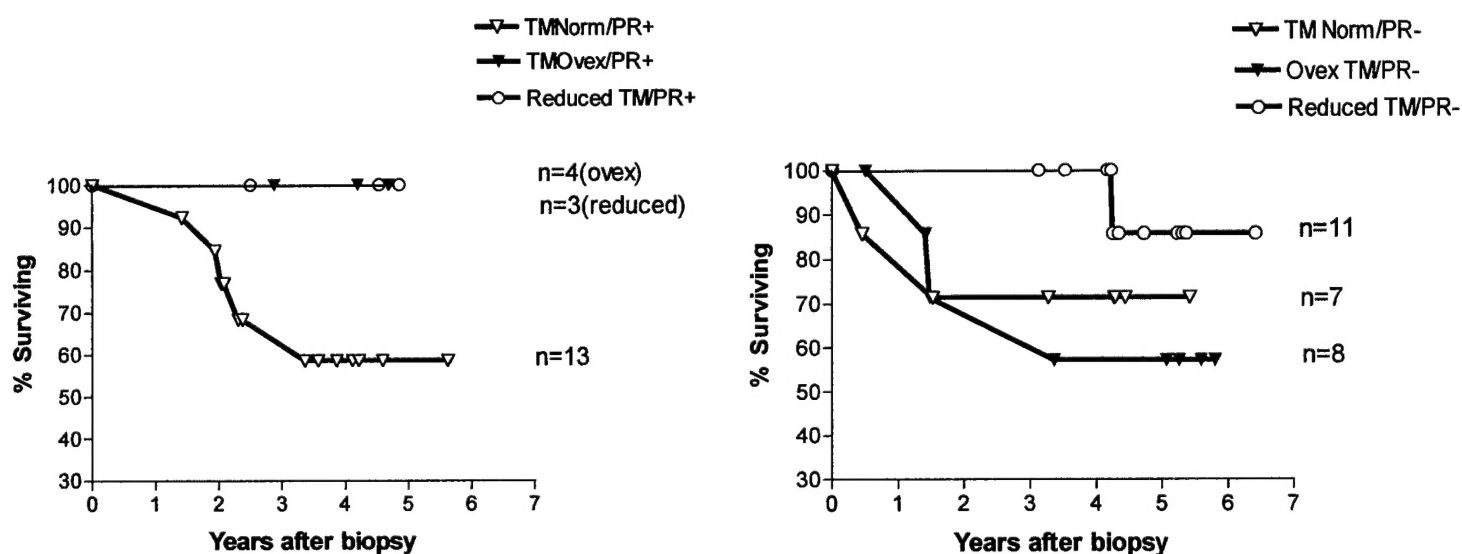


FIGURE 7. Survival analysis of breast cancer patients with PR+ and PR- tumors that had different levels of HMW TM expression. A Kaplan-Meier plot of survival using follow-up data from patients whose tumors were of the indicated PR status and had the indicated level of HMW TM expression. Life table analysis was as in Fig. 6. Differences in survival within the groups were not significant (PR+, $p=0.1991$; PR-, $p=0.2823$)